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Study of the human papillomavirus type 18 stable maintenance

Master`s thesis

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ABBREVIATIONS

ATP - Adenosine triphosphate

BPV - bovine papillomavirus

ccc - covalently closed circular form of DNA

CDK - cyclin-dependent kinase

CMV - Cytomegalovirus

Ct- qPCR cycle threshold value

DDR - DNA damage responce

DMSO - dimethyl sulfoxide

dsDNA - double-stranded DNA

DTT - dithiothreitol

E2F - a group of trancription factors in higher eukaryotes

EDTA buffer - ethylenediaminetetraacetic acid buffer

EGFR - Epidermal growth factor receptor

EtOH - ethanol

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

HA - hemaglutinin

HPV - human papillomavirus

HR - homologous recombination

HR-HPV - High-Risk HPV

HRP- horseradish peroxidise

IMDM - Iscove`s Modified Dulbecco`s Medium

kbp - kilo base pairs

LB - lysogeny broth

lin - linear form of DNA

LR-HPV - Low-Risk HPV

mc - minicircle

MHC - major histocompatibility complex

nt - nucleotide

oc - open circular form of DNA

ORF - Open Reading Frame

Ori - origin of replication

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PI - Propidium Iodide

pRb - retinoblastoma protein

PV - papillomavirus

PVDF - polyvinylidene difluoride

qPCR - quantitive PCR (real-time PCR)

rDNA - ribosomal DNA

SDS buffer - sodium dodecyl sulphate buffer

SSC buffer - saline-sodium citrate buffer

TAE buffer - Tris-acetate-EDTA buffer

TB - terrific broth

TBST- Tris buffered saline with Tween 20

TE buffer - Tris-EDTA buffer

TF - Transcription Factor

U2OS - human osteosarcoma cell line

URR/LCR - Upstream Regulatory Region/Long Control Region

wt - wild-type

INTRODUCTION

Papillomaviruses (PV) represent diverse group of DNA viruses with oncogenic potential. They infect the skin and mucosal epithelium of mammals, birds and reptiles. More than 170 human papillomaviruses (HPV) have been characterized so far, and infection with the majority of them causes benign warts or papillomas. Most infections are cleared by the immune system in approximately 24 months. However, a small fraction of infections could become persistent and develop into malignant tumors. Infection with high-risk HPV is responsible for almost all cases of cervical cancer and nearly 270 000 deaths annually.

The HPV life cycle is strictly dependent on the keratinocyte differentiation, and relies mostly on the host`s replication machinery. Three phases of HPV replication are distinguished. After entering the nucleus, the first (initial) viral genome amplification occurs, during which the viral DNA is replicated up to 50-100 copies per cell. The stable replication, or latency, follows next, where HPV genomes are replicated once per cell cycle, and viral copy number remains constant. The last stage is vegetative amplification of viral DNA that takes place in differentiated keratinocytes, during which HPV genome copy number is rapidly replicated up to a few thousand copies per cell. U2OS cells used in this work allow to study all replication stages of different HPVs. In the present study, the role of HPV18 early proteins during initial amplification and stable replication is studied.

The understanding of which viral proteins are necessary at different stages of HPV replication could be used to develop chemical compounds that inhibit viral replication.

1. LITERATURE OVERVIEW

1.1. Human papillomavirus

Papillomaviruses (PV) are a group of small double-stranded (ds) DNA tumor viruses that belong to the Papillomaviridae family. PVs posess high species specifity and can infect mammals, birds and reptiles (Bernard et. al., 2010). There are more than 200 PV types described today, and approximately 170 of them infect humans (Bernard et al., 2013; de Villiers, 2013; http://www.hpvcenter.se/html/refclones.html). Due to the high tissue specificity, they can only infect undifferentiated keratinocytes of stratified epithelium. Human papillomaviruses (HPV) infect human cutaneous or mucosal epithelia (Jenson et al., 1985; zur Hausen, 2002). Depending on their oncogenic potential, the mucosal HPVs are categorized into two groups: low-risk (LR) and high-risk (HR) HPV types. LR-HPVs cause benign warts and papillomas, but infection with HR-HPVs, such as HPV16 and HPV18, can progress to malignant tumors, most prevalent of which are cervical carcinomas (zur Hausen, 2009). According to the World Health Organization, every year there are estimated 528 000 new cases of cervical cancer worlwide, and 266 000 cases end with death, thus making it the second most common cancer among women. And more than 70% of the cases are caused by HPV16 and 18 alone (Li et al., 2011; http://globocan.iarc.fr; http://www.who.int/en/). However, not all infections with HR-HPVs lead to the malignancies. The majority of them are cleared within 24 month, while up to 20% develop into persistent infection that can last for years and progress into cancer (Stanley, 2008).

1.2. HPV genome

HPVs are small, non-enveloped viruses, 55-60 nm in diameter with icosahedral capsid. The HPV genome is a circular dsDNA molecule of about 8kbp, that is associated with histones (Favre et al., 1977). Different types of HPV have similar genome organization. All virus genes are located on a single DNA strand and transcribed as polycistronic mRNAs (Remm et al., 1999; Rotenberg et al., 1989). The genome is divided into three functional regions: the long control region (LCR), the early (E) and the late (L) regions (*Figure 1*). The LCR, or the upstream regulatory region (URR), is a non-coding region which consists of sequences that regulate the expression of viral genes, viral genome replication and its packaging into virus particles. It contains the replication origin, promoters, enhancers, silencers, and multiple binding sites for cellular transcription factors (TF) as well as for viral proteins E1 and E2

(Bernard, 2013). The early region has up to eight open reading frames (ORF), depending on the HPV type: E1-E8 ORFs. They are expressed in the lower epithelial layers and encode proteins involved in virus replication, transcriptional regulation, oncogenesis and cell cycle regulation. The late region contains two ORFs, L1 and L2, that encode for major and minor viral capsid proteins, which are expressed in the upper layers of the epithelium, in terminally differentiated keratinocytes (Burd, 2003; McBride, 2008; Orth, 2008; Zheng & Baker, 2006).

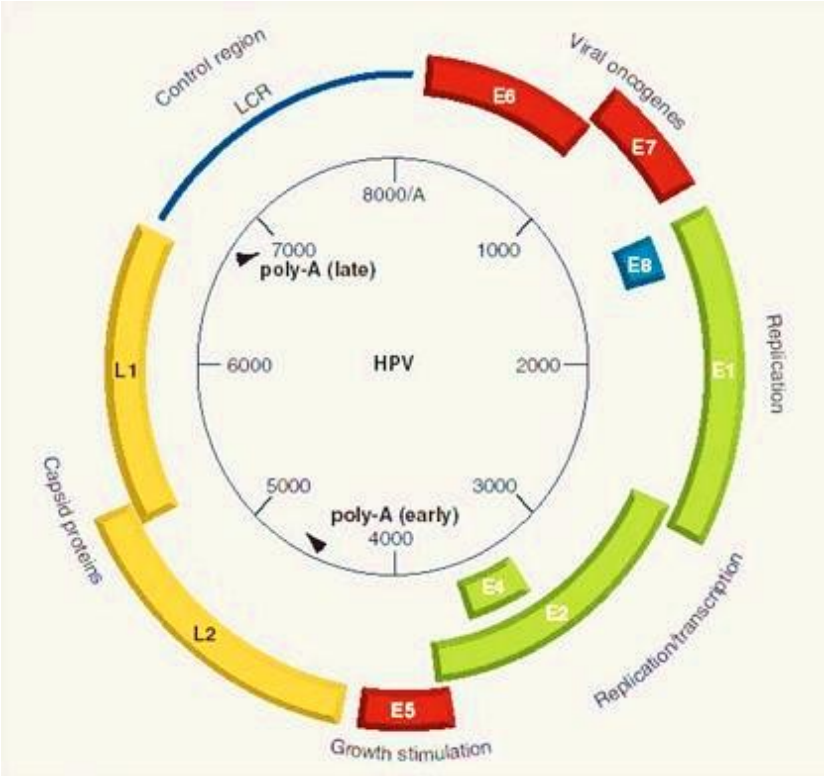

Figure 1. The HPV genome organization. Three regions of HPV genome are shown: the upstream LCR region, which contains viral replication origin and transcriptional regulatory elements; the early region, encoding for early expressed proteins E1, E2, E4, E5, E6, E7 and E8; the late region, encoding for capsid proteins L1 and L2. Two polyadenylation sites separate three regions. (Adapted from Lie and Kristensen, 2008).

1.3. Functions of HPV proteins

Early proteins.

The E1 protein is the most important and essential viral protein for HPV genome replication. It is an ATP-dependent DNA helicase that interacts with the E2 protein, which traffics it to the origin of replication in the URR. Thus, E1 initiates the HPV replication, by melting and

unwinding the viral DNA (Chiang et al., 1992; del Vecchio et al., 1992; Jenkins et al., 1996; Sedman and Stenlund, 1998). Also, E1 interacts with many cellular replication factors, like polymerase, topoisomerase, replication protein A, and brings them to the viral replication site, thus inducing them into viral genome replication (Clower et al., 2006; Conger et al., 1999; Loo and Melendy, 2004).

The E2 protein is a multifunctional site-specific binding protein, that attaches to the LCR of the HPV, and regulates viral replication and transcription. It is essential in the replication initiation as it forms complex with the E1 protein and loads it onto the origin of replication (Chiang et. al., 1992; Stenlund, 2003). E2 is a major transcription regulator of viral genes. Cooperating with cellular TFs, it can act as viral transcription activator or repressor. It has been shown that E2 can supress the transcription of E6 and E7 genes (Bouvard et al., 1994; Chin et al., 1988; Schweiger et al., 2007; Steger and Corbach, 1997). In addition to transcriptional regulation, E2 is responsible for viral genome segregation as well. E2 tethers the viral genome to the host chromosomes, and ensures partitioning of the replicated viral DNA to the daughter cells (Bastien and McBride, 2000; Skiadopoulos and McBride, 1998).

E1 and E2 proteins have shown to cause chromosomal instability as they facilitate the replication of the integrated HPV DNA, what induces the activation of DNA damage responce (DDR) (Kadaja et al., 2007; 2009).

In addition to the full-length form of E2, PVs also express its short variant E8^E2, a fusion protein encoded by spliced mRNA that fuses the small E8 ORF and the C-terminal DNA binding/dimerization domain of E2. The E8^E2 protein acts primarily as viral transcription and replication repressor through interaction (by E8 domain) with different cellular factors, by competing with the full-length E2 for binding to the URR region and by forming heterodimers with E2 protein (Ammermann et al., 2008; Kurg et al., 2010; Stubenrauch et al., 2001; Wang et al., 2011; Zobel et al., 2003). Although the transcriptional repression by E8^E2 of viral early promoter and replication inhibition in undifferentiated cells was shown, recently Straub et al. managed to demonstrate that E8^E2 has a repressive influence on the whole productive life cycle of HPV16. It’s role in stable mainetance of HPVs, however, is still unclear. It has been shown that E8^E2 is dispensable for stable maintenance of HPV16 and HPV18.

However it seems to be important protein for HPV31 stable replication. (Kurg et al., 2010; Lace et al., 2008; Straub et al., 2014; Stubenrauch et al., 2000).

The E4 protein is mostly expressed as a E1^E4 fusion protein, a product of spliced mRNA of the E1 ORF`s first five amino acids and the whole E4 ORF (Nasseri et al., 1987; Wang et al., 2011). The E1^E4 is synthesized at very high levels in the mid and upper, most differentiated layers of epithelum at the late stages of productive infection (Doorbar et al., 1997). It takes part in viral genome amplification and the expression of late genes, thus having a supportive role in HPV vegetative replication (Doorbar, 2013; Nahakara et al., 2005; Wilson et al., 2007). E1^E4 is strictly cytoplasmic protein. It interacts with cellular cytoskeletal keratin filaments and disrupts the cytokeratin network. In addition, it causes morphological changes in virus-laden cornified cell envelopes. All this properties, as well as the ability to induce apoptosis through binding to the mitochondria, are likely to facilitate the release of viral particles from the cell (Brown, et al., 2006; Doorbar et al., 1991; 1997; Raj, et al., 2004; Wang et al., 2004).

Oncoproteins.

The E5 protein is a small hydrophobic oncoprotein that is located near endosomal and cellular membranes and in close approximation to the Golgi apparatus (Conrad et al., 1993). Expressed alone E5 has a week transorming activity, while its co-expression with E6 and E7 proteins facilitates the carcinogenesis at more higher levels than with either of the proteins alone (Stöppler et al., 1996). E5 enhances cell proliferation by interfering with different cellular factors, like through increasing the level of the epidermal growth factor receprors (EGFR) or by disrupting the tumor suppressor proteins p21 and p27 (Pedroza-Saavedra et al., 2010; Straight et al., 1993; Tsao et al., 1996). E5 helps to escape the host immune system by down-regulating the expression of major histocompatibility complex (MHC) I and MHC II in infected cells (Ashrafi et al., 2006; Zhang et al., 2003). Also, E5 is capable of inhibiting the cell apoptosis, thus promoting cell survival and transformation which is necessary for HPV to replicate its genome (DiMaio and Petti, 2013; Kabsch and Alonso, 2002; Oh et al., 2010; Zhang et al., 2002).

The E6 and E7 proteins are multifunctional oncogenes, that are causative agents of HPV-associated tumors (Münger, 2002; Roman and Munger, 2013; Vande Pol and Klingelhutz, 2013).

The main function of the E6 protein is the degradation of cell tumor-suppressor protein p53 through the ubiquitin-proteasome pathway. This feature, common for all HR-HPV types, helps to avoid the apoptosis of the infected cell and can lead to its transformation. However, E6 can also block apoptosis in p53-independent manner (Fu et al., 2010; Howie et al., 2009; Scheffner et al., 1990). Also, E6 promotes the cell immortalization by activation of telomerase thus preventing shortening of telomers and expanding the keratinocytes` lifespan (Klingelhutz et al., 1996). Among another functions of E6 that contribute to HPV carcinogenesis are disruption of cell adhesion and polarity, inhibition of keratinocytes differentiation, decrease of the immune response, bypassing of G1/S and G2/M damage-induced checkpoints and reducing genomic stability (Klingelhutz and Roman, 2012; Pang and Thierry, 2013).

The main feature of the E7 protein is its ability to interact with retinoblastoma tumor suppressor protein (pRb) and its family proteins p107 and p130. pRb in complex with cellular TFs (E2F) acts as a repressor in the G1/S transition. So, E7 association with active hypophosphorylated pRb form disrupts the pRb/E2F complexes, and, as a result, allows the cells to re-enter the S phase and to synthesize DNA (Dyson et al., 1989; Jones and Münger, 1997; Münger, 2001). Besides, E7 targets the pRb and its family proteins for ubiquitin-proteosome degradation (Wang et al., 2001). It has been also shown that E7 can overcome the growth arrest by direct binding to the cyclin-dependent kinase (CDK) inhibitors (Funk et al., 1997; Zerfass-Thome et al., 1996). All these features, as well as the cellular genome instability caused by centrosome abnormalities induced by E7, contribute to HPV carcinogenesis (Duensing et al., 2001).

Structural proteins.

Two capsid proteins, L1 and L2, are synthesised at the last stage of viral infection in differentiated keratinocytes. L1 is a major capsid protein and it is responsible for capsid formation by making 72 joined together pentamers (Buck and Trus, 2012). This ability of L1 is used in producion of DNA-free virus-like particles (VLPs), that are used in preventive vaccination against HPVs (Kirnbauer, 1992; FDA 2006; 2009). Also, L1 is essential in virus

entry into the host cell by interacting with cell receptors (Culp et al., 2006; Johnson et al., 2009; Joyce et al., 1999). L2 is a minor capsid protein, every single molecule of it associates with one L1 pentamer (Buck et al., 2008; Buck and Trus, 2012). It has important role in viral DNA encapsidation, the endocytosis after cell entry and transport of viral genome to the nucleus (Day et. al., 2004; Kämper et al., 2006).

1.4. HPV life cycle

The HPV lifecycle and replication is strictly dependent on keratinocyte differentiation. Virus infects the host organism through microwounds in the epithelium and targets only mitotically active undifferentiated basal cells (Kines et al., 2009; Pyeon et al., 2009). Virus enters the cell by endocytosis and moves to the nucleus where it replicates using mostly the host replication machinery. Three stages of HPV genome replication are distinguished: the initial amplification, the stable replication (stable maintenace), and the final (vegetative) amplification (*Figure 2*).

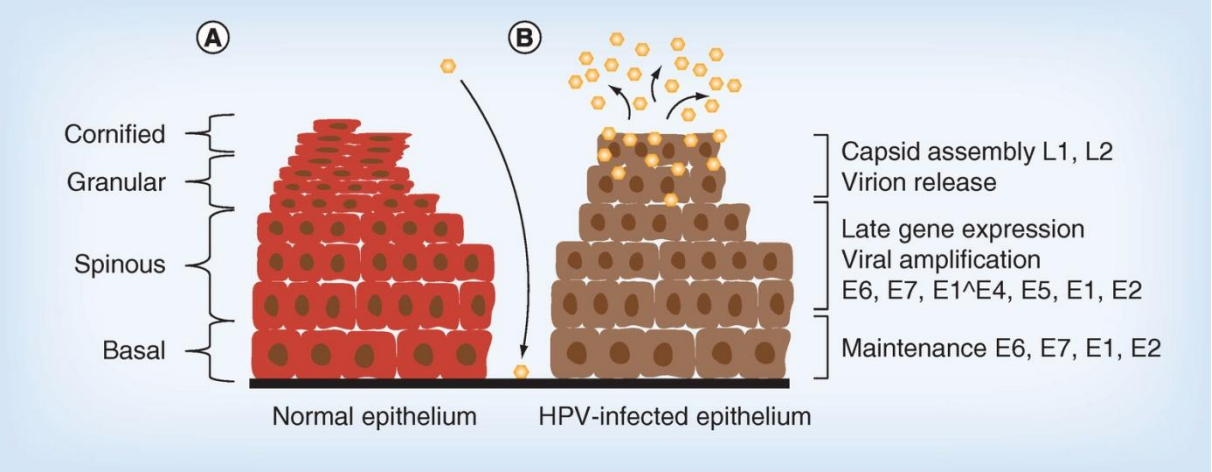

Figure 2. HPV productive life cycle. HPVs infection occurs through microabrasions in the epithelium where virus targets basal layer keratinocytes. (A) represents a normal uninfected epithelium. (B) represents the productive HPV infection. After the nucleus entry, the viral genome initial amplification takes place with the following viral genome maintenance phase. The viral protein expression occurs only at a low level. As keratinocytes differentiate, HPVs move together with the cells upward the epithelium, where viral vegetative amplification is triggered. HPV late genes are expressed, new virions are assembled and released from cell with the epithelium desquamation. (Adapted from Hong and Laimins, 2013).

The initial amplification.

Following entering the nucleus, the establishment of viral genomes as extrachromosomal episomes occures. For the replication initiation, E1 and E2 proteins form a complex and bind

to the replication origin in the URR region. After binding, the E1 hexameric complex is formed, E2 is released, and cellular replication factors are transfered to the origin of replication. Working as helicase, E1 separates two viral DNA strands in front of the replication complex (Chiang et al., 1992; Sedman and Stenlund, 1998; Stenlund, 2003). It is considered that during transient replication E1-dependent HPV genome replication takes place according to the bidirectional theta replication model. The genome amplifies several times per cell cycle thus reaching 50 to 100 copies per cell in a short period of time (Doorbar, 2005; Flores and Lambert, 1997).

It has been shown that during initial amplification HPVs trigger DDR. The exact mechanism and benefit of this activation is not clear, propably virus uses it to promote its survival and replication. When DDR is activated, large amounts of cellular proteins involved in DNA repair and replication are localized to the damaged sites. Thus, HPVs could use these proteins to efficiently synthesise its DNA. Moreover, it has been suggested that besides bidirectional theta model, HPVs use homologous recombination (HR; important pathway in error-free DNA repair) dependent replication during initial and vegetative amplification (Fradet-Turcotte et al., 2011; Gillespie et al., 2012; Moody and Laimins, 2009; Orav et al., 2013; Reinson et al., 2013; Sakakibara et al., 2011; 2013).

The stable replication/The stable maintenance.

For the persistent HPV infection, the viral genome must be maintained, stably replicated and segregated during the cell devision. This stage continues in basal cells, where the HPV genome has established, and may last for years with minimal gene expression pattern to avoid host immune response. During this phase viral genome replicates once per cell cycle with constant low copy number (up to few hundred copies) per cell (Doorbar, 2005; Hoffman et al., 2006). Most probably, as in initial viral genome establishment, during maintenance HPV also replicates according to the theta replication model (Flores and Lambert, 1997).

It has been considered that E1 and E2 proteins are both necessary during the whole life cycle of PVs. However, the study of BPV-1 maintenance and recent research done on HPV16 by Egawa et al. indicate that E1 might not be required for the stable replication (Egawa et al., 2012; Kim and Lambert, 2002). Though, it seems that E2 protein is essential for viral genome maintenance as it partitions it into daughter cells (Bastien and McBride, 2000).

It has been shown that E6 and E7 proteins are essential during stable replication for HPV maintenance as extrachromosomal episomes. Though during this phase they also induce

DDR, it does not play such crucial role as for vegetative replication (Lorenz et al., 2013; Moody and Laimins, 2009; Thomas et al., 1999).

HPV vegetative replication.

As the final aim of HPV productive life cycle is the formation of infectious virions, the virus must amplify its genome and package it into the capsid for the future release from the cell. The third phase of HPV genome replication starts as the keratinocytes undergo differentiation. And as the cells migrate up through the epithelium layers, the viral proteins are synthesised at very high levels, and the viral DNA amplification to many thousands of copies per cell occurs (Bedell et al., 1991; Doorbar 2005). In a healthy epithelium, differentiating keratinocytes exit cell cycle and sometimes lose their nuclei (Pang et al., 1993). As HPV replication is dependent on the host cellular factors that are synthesised only in mitotically active cells, the virus has to overcome this problem by the actions of E6 and E7 proteins. E7 represses the pRb family protein p130 function and thus makes cells to re-enter the S phase and to synthesise the cellular replication factors needed for HPV replication (Cheng et al., 1995; Zhang et al., 2006). HPV E6 protein binds p53 and targets it for degradation (Thomas and Chiang, 2005). During this stage, HPVs cause prolonged G2 phase. It has been shown that besides S-phase, HPVs could also replicate in G2 phase of the cell cycle, probably by inducing DDR response and using cellular DNA repair machinery. It has been shown that E7-dependent DDR activation is essential for vegetative amplification (Banerjee et al., 2011; Moody and Laimins, 2009; Reinson et al., 2013; Wang et al., 2009). Replication in G2 phase might be necessary because HPVs „need more time“ to achieve high viral copy numbers.

It has been suggested that during the vegetative replication, the bidirectional theta mode replication of HPV in undiffirentiated cells switches to an alternative mode. It is not clear how viral genome amplifies exactly, but after studying the replication intermediates, two ways have been proposed: a rolling-circle replication or a recombination-dependent replication (Dasqupta et al., 1992; Flores and lambert, 1997; Sakakibara et al., 2013).

After the vegetative replication, the synthesis of the late capsid proteins, L1 and L2, begins in the terminally differentiated keratinocytes. This is followed by the viral DNA encapsidation, and the assembly of E1^E4 protein into amyloid fibers which disrupts cell keratin and facilitates virions release with the epithelium desquamation (Mcltosh et al., 2008; Wang et al., 2004).

1.5. U2OS cell line

The human osteosarcoma U2OS cell line was used in this study. It is an easy and cost-effective system to study HPV genome replication. It can be used to study all three stages of the replication of HPVs infecting mucosal and cutaneous epithelia. Initial amplification occurs after transfection of HPV DNA into the cells. This stage occurs for approximately one week after the transfection. Stable replication can be studied by selecting the transfected cells and by culturing them as subconfluent culture. Vegetative amplification is turned on by culturing the selected cells in confluent conditions for at least 10 days (Geimanen et al., 2011). This immortalized cell line was derived from a bone tumor of a 15 years old female. U2OS cells are morphologically similar to the epithelial cells, and carry a wild-type (wt) p53 and pRb genes (Ponten and Saksela, 1967).

2. EXPERIMENTAL STUDY

2.1. Aims of this study

The aim of the present study is to describe the HPV18 initial amplification and stable maintenance in the U2OS cell line and to identify which HPV18 gene products are necessary for viral genome replication during this phase.

2.2. Materials and methods

2.2.1. Plasmids

pM18-1.4 – HPV18 E1 protein expression vector, that contains HPV18 E1, E6 and E7 ORFs cloned into QM – Ntag/Ai+ vector. Cytomegalovirus (CMV) promoter lies before E1 ORF. Hemagglutinin (HA) tag is added at the beginning of E1 ORF.

pQMN18-E2 – HPV18 E2 protein expression vector, that contain HPV18 E2 ORF that was cloned into QM – Ntag/Ai+ vector. CMV promoter is situated before E2 ORF.

pMC.BESPXHPV18 – HPV18 minicircle parental plasmid. To construct this plasmid, restriction enzyme BglII site was introduced into the HPV genome after nucleotide (nt) 7473 and used for cloning into pMC.BESPX vector. All HPV18 genomes were cloned based on this plasmid.

HPV18 wt minicircle – wild-type HPV18 genome sequence.

HPV18E1^E4 mutant – stop codon was made after the splice site at nt position 3434 by mutations at nt position 3452, A→G, and T→A at nt position 3453.

HPV18E4 mutant – at the nt position 3419, the ATG start codon was changed to ACG**.**

HPV18E5 mutant – from the nt position 4011, nucleotides TAACTCGAG were added.

HPV18E6 mutant – from the nt position 134, nucleotides CTCGAGG were added.

HPV18E7 mutant – from the nt position 610, nucleotides TCGGAGAAC were added.

HPV18E6E7 mutant – from the nt position 134, nucleotides CTCGAGG were added, and from the nt posistion 610 were added TTAGTCGACAAC nucleotides.

HPV18E8 mutant – at the nt position 1324, the ATG start codon was changed to ACG

HPV18E2C-2 mutant – at the nt positions 3426, ATG start codons were changed to GCG.

HPV18E8^E2C-1 mutant – at the nt positions 1324 and 3253, ATG start codons were changed to, accordingly, ACG and GCG.

HPV18E8^E2C-2 mutant – at the nt positions 1324 and 3426, ATG start codons were changed to, accordingly, ACG and GCG.

pBabeNeo – mammalian expression vector containing neomycin resistance marker.

E1 siRNA – small interfering RNA molecule designed against region 965-987 in HPV18 genome, which action results in the E1 mRNA degradation.

2.2.2. pBabeNeo plasmid linearization and purification

To linearize pBabeNeo plasmid, a reaction mix with restriction enzyme EcoRI was prepared and incubated at least 1 hour at 37°C. Linearized pBabeNeo plasmid was purified using NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel) according to the manufacturer`s protocol.

2.2.3. HPV18 minicircle production

Minicircles are recombinant covalently closed circlular DNA vectors that are devoid of procaryotic elements. The HPV18 parental minicircle plasmid was constructed by inserting the pMC.BESPX minicircle vector into the HPV18 genome between nt 7473 and 7474 (Kay et al., 2010; Orav et al., 2014; Reinson et al., 2013). In this parental plasmid, different frameshift- or pointmutations were introduced into the gene of interest, so the functional protein was not translated. For the present work, altogether, 11 different HPV18 minicircle genomes were constructed: HPV18wt, HPV18 E1^E4$^-$, E4$^-$, E5$^-$, E6$^-$, E7$^-$, E6$^-$E7$^-$, E2C-2$^-$, E8$^-$, E8$^-$E2C-1$^-$ and E8$^-$E2C-2$^-$. These mutants were constructed by Mart Toots and Tormi Reinson.

For the production of minicircles, Escherichia coli ZYCY10P3S2T strain was transformed by heat shock method. 0.5µg of plasmid DNA were mixed with competent bacterial cells (200µl), held on ice for 15min, then incubated for 3min at 37°C and cooled on ice for 1min. After, 850µl of the lysogeny broth (LB) were added to the cells and the 30min incubation at 37°C followed. Then, the mixture was centrifuged for 1min at 4000g, cells were resuspended in 100µl of LB and plated on Kanamycin (50µg/ml) containing agar plates. The overnight incubation at 37°C followed. One bacterial colony was picked up and inoculated into 3ml of LB medium and grown in a shaking incubator (220rpm) at 37°C for 6 hours. Then, 200µl of starter culture were added to 100ml of Kanamycin (50µg/ml) containing terrific broth (TB) and grown overnight at 37°C with shaking at 220rpm. To induce the site-specific recombination, 100ml of induction mix (0.04M NaOH, 0.04% L-arabinose in LB medium) were added and the culture was incubated for 8 hours at 32°C, shaking at 220rpm. Bacteria were collected by centrifugation at 4000g, 4°C for 15min and stored at -20°C. Minicircle plasmids were purified using NucleoBond® PC 500EF Kit (Machery-Nagel) according to user`s manual. To ensure that the purified DNA is strictly covalently closed circular minicircles, it was analyzed on 0.8% agarose gel, in 1xTris-acetate-EDTA (TAE) (40mM Tris-acetate, 1mM EDTA) buffer. Gel electrophoresis was carried at 5V/cm for 30min.

2.2.4. Cell line

Human osteosarcome cell line (U2OS) was used in present work. Cells were grown in Iscove`s Modified Dulbecco`s medium (IMDM) with the addition of 10% fetal bovine serum and antibiotics Penicillin (100U/ml) and Streptomycin (100µg/ml). Cells were cultivated in incubator at 37°C with CO_2 level of 5%.

2.2.5. Transfection of U2OS cells by electroporation

Each transfection was performed using one subconfluent 100mm cell culture dish (U2OS, density 80%-90%). The growth media was removed and cells were washed with 1xPBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4). Cells were detached with 2ml of trypsin/EDTA and collected into one tube that contained as much media as the total amount of used trypsin. Cells were harvested by centrifuging 5min at 300g (Eppendorf Centrifuge 5810 R). After, cells were resuspended in the growth media (250µl for one transfection). Next, cells and DNA were mixed in an electroporation cuvette and transfected using Bio-Rad GenePulser Xcell II apparatus (Bio-Rad Labaratories) at 220V, 975µF. 200µl of media were added into cuvette, and cell suspension was transfered into a 15ml tube, which contained 2ml of growth media. Then cells were centrifuged at 300g for 5min. All transfected cells were taken in 1ml media and plated onto 60ml dishes for future analysis.

2.2.6. Chemical transfection of U2OS cells stably maintaining HPV18 wt genome

Each transfection was performed using one 60mm cell culture dish. The R007 (Icosagen Cell Factory OÜ) reagent was used to mediate the transfection. The first step was the formation of the R007/DNA or R007/siRNA complex with the proportion of 1µg of DNA or 150pmol of siRNA to 7.6µl of R007 (400µM) working solution, reaction volume was taken to a total of 50µl with MQ water. According amount of DNA or siRNA was mixed up with MQ water by vortex, and then R007 was added and carefully suspended by pipetting. For the formation of siRNA/DNA-protein complex the solution was incubated at the room temperature (RT) for 1 hour. During this, the cells were washed with growth medium and 3ml of new medium were added. The transfection mixture was added to the media and cells were incubated at 37°C with CO_2 level of 5% until analyzed.

2.2.7. Total DNA extraction

The media was removed and cells were washed twice with 1xPBS. Cells were lysed with 0.5ml of 20mM Tris-HCl pH 8.0, 100mM NaCl, 10mM EDTA, 0.2% SDS and Proteinase K (0,2µg/µl) mixture. The cell lysate was transfered into 2ml tubes, pulled through the syringe for three times, and incubated at 37ºC for at least 18 hours. Equal amount of phenol-chloroform mixture (1:1) was added into every tube to separate DNA from proteins. Tubes were centrifuged at 16060g for 2min. The supernatant was transferred to a new tube and DNA was precipitated with two volumes of 96% ethanol (EtOH). The 30min incubation at -20ºC and afterward centrifugation at 16060g for 10min at 4ºC followed. The DNA was then dried and redissolved in 300µl of Tris-EDTA (TE) (10mM Tris-HCl pH 8.0, 1mM EDTA) buffer containing RNaseA (20µg/ml), and incubated at 37ºC for 1 hour. The DNA was precipitated with 12µl of 5M NaCl and two volumes of 96% EtOH and incubated at -20ºC for 30min. Then the precipitant was centrifuged at 16060g and 4ºC for 10min, washed with 700µl of 70% EtOH, dried and dissolved in 50µl of TE.

DNA concentrations were measured with NanoDrop Spectrophotometer ND1000. Samples were cleaved overnight at 37ºC using either BglI, which linearizes HPV DNA, or HindIII which cleaves only cellular DNA. For transient replication assay, DNA was also digested with DpnI enzyme to distinguish between transfected and newly synthesised DNA**.**

2.2.8. Cell lysis for Western blot

The media was removed and cells were washed with 1xPBS. Cells were detached with 1ml of PBS-3mM EDTA by incubating for 5 min at room temperature and then transfered into 1.5ml tubes. The centrifugation at 855g for 2 min followed. The supernatant was removed and cells were resuspended in 40µl of 1xPBS and lysed with 40µl of 2× Laemmli loading buffer (65.8mM Tris-HCl pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) containing 200mM DTT. The probes were boiled at 100ºC for 10 min. Before gel electrophoresis the samples were centrifuged at 16060g for 5 min.

2.2.9. Western blot

The proteins were separated on 10% polyacrilamide gel (SDS-PAGE) in 1xTris-Glycine-SDS buffer (25mM Tris, 192mM glycine, 0.1% SDS pH 8.3). Gel electrophoresis was carried at 120V-180V. Proteins were transfered into polyvinylidene difluoride (PVDF) membrane (Immobilon®-P, Millipore) using Trans-Blot SD, Semy-Dry transfer cell apparatus (BioRad) at 15V for 30min. After transfer, the membrane was blocked in 5% dry milk containing TBST

buffer (50mM Tris-HCl pH. 7.5, 150mM NaCl, 0.1% Tween 20) overnight at 4°C. Next, the membrane was incubated for 1 hour at RT with primary antibody in 3% dry milk in TBST. The membrane was washed 3x5 minutes with TBST and incubated for 1 hour at RT with the secondary antibody conjugated with HRP (horseradish peroxidise in the 3% blocking buffer. After washing for 3x5 minutes with TBST, the proteins were visualized using ECL™ kit and exposed to X-Ray film.

Antibodies used:

- Rat monoclonal anti-HA-tag antibody 3F10 conjugated with HRP (Roche), dilution 1:1000
- Mouse monoclonal beta-Tubulin antibody (Sigma T8328), dilution 1:8000
- Goat Anti-Mouse HRP-conjugated antibody (LabAS), dilution 1:8000

2.2.10. Southern blot

Extracted genomic DNA was resolved in 1% agarose gel in 1xTris-acetate-EDTA (TAE) (40mM Tris-acetate, 1mM EDTA) buffer. Gel electrophoresis was carried at 0,5V/cm overnight. The DNA in the agarose gel was denatured by incubating the gel in Sol A (0.5M NaOH, 1.5M NaCl) buffer for 30 min. The gel was then rinsed with distilled water and neutralized with Sol B (1M Tris, pH 7.4, 1.5M NaCl) buffer for 30 min. The DNA was transferred overnight by capillary transfer to a nylon membrane (Membrane Solutions) using 10×saline-sodium citrate buffer (SSC; 1,5M NaCl, 150mM Na-citrate) buffer. After, the DNA was cross-linked to the nylon memrane with UV Stratalinker 1800 (Stratagene). Then, to avoid non-specific binding to the membrane, it was treated with prehybridization solution (30% 20×SSC, 10% 50×Denhardt's, 5% 10% SDS, 2% 10mg/ml carrier DNA) for 45 min at 65°C.

The HPV18 specific probe was labeled with radioactive [α-^{32}P]-dCTP using a DecaLabel™ DNA Labeling Kit (Thermo Scientific). 100ng of linearized HPV18 genome, 10 µl of decanucleotides in 5x reaction buffer and 28µl MQ water were mixed, incubated for 10 min at 100°C and cooled on ice. Then 3 µl of the nucleotides` Mix C, lacking 2'-deoxycytidine 5'-triphosphate (-dCTP), 1 µl of Klenow Fragment, *exo-*, enzyme with polymerase activity and 60µCi of [α-^{32}P]-dCTP isotope were added, and the probe was incubated at 37°C for 10 min. 4 µl of dNTP solution were added and probe was incubated at 37°C for 10 min. The reaction was stopped by the addition of 1 µl of 0,5M EDTA and incubation at 100°C for 10 min. The radiolabeled probe was added to the prehybridization solution, and the filter was hybridized

overnight at 65°C. After hybridization, the membrane was washed with three different solutions:

1. 2 x 5 min with buffer I (2×SSC, 0,1% SDS)
2. 15 min with buffer II (1xSSC, 0,1% SDS)
3. 2 x 10 min with buffer III (0,1xSSC, 0,1% SDS).

The signal were detected using phosphoimager (Typhoon TRIO, Amersham Bioscience).

2.2.11. Real-time quantitative PCR

HPV18 copy number quantification in U2OS cells was performed using real time quantitive PCR (qPCR). Linearized and restricted DNA (50-125ng) was diluted in 200µl MQ water.

The qPCR was carried in 10 µl volume:

- 2 µl 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (SolisBiodyne)
- 0,3 µl F primer (10µM)
- 0,3 µl R primer (10µM)
- 2,4 µl MQ water
- 5 µl template DNA solution (1,25-3,125ng)

The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene or ribosomal DNA (rDNA) was used as a reference gene. For DNA amplification 300nM of each forward and reverse primers were used (*Table 1*)

Table 1. Primers for qPCR.

HPV18 E7, F	5'-GCGCTTTGAGGATCCAAC-3'
HPV18 E7, R	5'-GTTCCGTGCACAGATCAG-3'
HPV18 URR, F	5'-AGGTTGGGCAGCACATAC-3'
HPV18 URR, R	5'-AGAAGACGTAGTGGCAGATG-3'
GAPDH, F	5'-TACTAGCGGTTTTACGGGCG-3'
GAPDH, R	5'-ACAGGAGGAGCAGAGAGCGA-3'
rDNA, F	5'-CCTGCGGCTTAATTTGACTC-3'
rDNA, R	5'-TCGCTCCACCAACTAAGAAC-3'

A 384-well plate was used and, during the sample loading process, kept on ice. Three parallels were run with each primer set, MQ was used as control. After the samples were loaded, the plate was sealed with optical adhesive film and centrifuged for 1min at 40g.

The Applied Biosystems 7900HT Fast Real-Time PCR System was used for DNA amplification with the following thermo cycle programme:

* PCR activation	15 min	95ºC	
* denaturation	15 sec	95ºC	x 40 cycles
* primer annealing	30 sec	60ºC	
* primer extention	30 sec	72ºC	
* dissociation stage		60 - 95ºC	

The qPCR data was analyzed using the Comparative threshold cycle (ΔΔCt) method. During relative quantitation, the amount of HPV18 as well as reference gene is determined from the cycle threshold values (Ct) using the SDS 2.2.4 programme (Applied Biosystems). The followed analysis was performed using Microsoft Excel (2007). The relative C_N value, which reflects an average viral genome copy number per cell, was calculated with the formula $C_N = 2^{-\Delta Ct}$, where ΔCt is the difference between threshold cycles of HPV18 and cellular GAPDH or ribosomal DNA. The fold difference of different samples was calculated by deviding the C_N of a sample to C_N value of the control sample that the rest were compared to.

2.3. THE RESULTS

2.3.1. HPV18 transient replication analysis

The U2OS cells were transfected by electroporation with 2µg of HPV18 different genomes together with 1µg of linearized pBabeNeo plasmid. pBabeNeo is a mammalian expression vector that carries a neomycin resistance marker, thus making it resistant to geniticin (G418) and allowing the selection of transfected cells. Transfected cells were plated on 60mm dishes for transient and stable replication analysis. For the selection control, the mock transfection was also carried. For transient replication analysis, cells were grown without selection. 3 and 5 days after transfection, genomic DNA was extracted and analysed by Southern blot (*Figure 3*). Before analysis, the 3µg of DNA were cleaved overnight with BglI to linearize HPV DNA, and with DpnI to distinguish between transfected and replicated DNA. Linearized DNA was resolved in agarose gel and transfered onto the nylon membrane. Hybridization with HPV18 specific radio-labeled probe and the signal detection with Typhoon Phosphoimager followed.

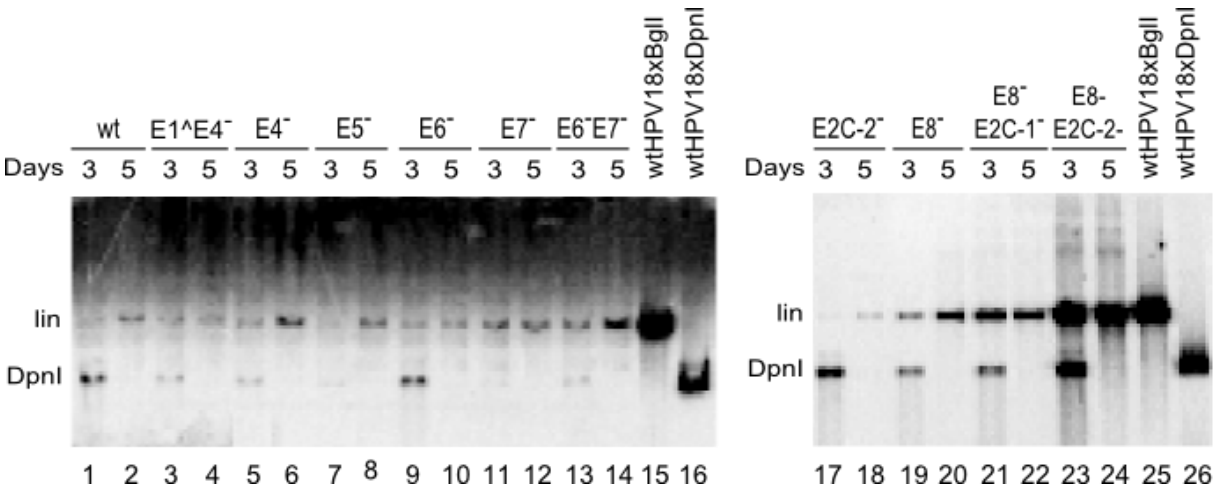

Figure 3. Transient replication analysis of HPV18 wt or its mutant genomes in U2OS cell line. HPV18 E1^E4$^-$, E4$^-$, E5$^-$, E6$^-$, E7$^-$, E6$^-$E7$^-$, E2C-2$^-$, E8$^-$, E8$^-$E2C-1$^-$ and E8$^-$E2C-2$^-$ genomes with introduced pointmutations or frameshift mutations were used. The U2OS cells were transfected with 2µg of minicircle DNA together with 1µg of the linearized pBabeNeo plasmid. Total DNA was extracted 3 and 5 days after transfection. DNA was digested with BglI enzyme to linearize viral genome, and with DpnI enzyme to distinguish between transfected and newly synthesised HPV DNA. The Southern blot analysis with HPV18 specific probe followed. Lines 1-14 and 17-24 represent the analysis of HPV18 wt and mutant genomes` replication signal at the indicated timepoints. Lines 15, 16, 25 and 26 represent the

size markers for linearized and DpnI restricted HPV18 genome. Signals for linearized replicated and DpnI restricted transfected HPV18 DNA are shown.

Figure 3 represents the Southern blot analysis of transient replication of HPV18 wt and mutant genomes in U2OS cells. The replication of all HPV18 genome mutants is clearly detected and it increases in time. Mutations in the E1^E4, E4, E5, E6 or E7 ORFs do not change the replication efficiency compared to the wt HPV genome (compare lanes 1 and 2 with lanes 3-14). Similalrly to previous studies (Kurg et al., 2010; Lace et al., 2008), E8 mutant (not expressing the repressor E8^E2) genome shows at least 5-fold higher replication compared to wt HPV18 (compare lanes 1 and 2 with lanes 19 and 20). Transcription analyses of HPV18 in U2OS cells identified promoter region in the ORF of E2 (P_{3000}; Männik et al., unpublished data), similar to the one described for BPV-1. This promoter could be used to encode regulators for HPV18 transcription and replication which contain only the C-terminal part of E2 protein (E2C; Choe et al., 1989; Lambert et al., 1987). For HPV18, ATG codons for two E2C proteins, E2C-1 and E2C-2, were identified. The mutation in the E2C-2 protein suprisingly decreases the replication compared to wt (*Figure 3*, lanes 17 and 18). However, HPV18 genomes bearing both E8 and E2C-2 or E8 and E2C-1 mutations show considerably higher replication compared to the genome containing only E8 mutation (*Figure 3*, lanes 19-24). This indicates that E8^E2 acts as primary regulator for HPV18 replication and E2C proteins have a minor role. All together this analyses shows that, apart from E1 and E2, none of the early gene products of HPV18 are essential for transient replication in U2OS cells. Transient replication takes place before stable maintenance phase. So, this experiment was necessary to see, whether these HPV18 mutants have the potential to remain stable in U2OS cells.

2.3.2. HPV18 stable replication analysis

The transfected cells from the same experiment shown in F*igure 3* were selected by adding the antibiotic G418 (400µg/ml) into the media. The media was changed after every two days during the experiment. The G418 concentration was decreased to 200µg/ml as the cells from the mock control (not transfected with pBabeNeo plasmid) died thus indicating that the rest cells cultivated under G418 were succesfully transfected. As the cells were confluent enough, they were transfered onto 100mm plates and cultivated to 80%-90% density. The whole process of rising drug-resistant HPV18 containing cell pools took more than 2 weeks. After the selection genomic DNA samples were collected after every fourth day during 2 weeks. Cells were maintained at subconfluent conditions during analyses. Thus, as a result, 4 passages (time points 0, 1, 2, 3) with cells carrying stably persistent HPV18 genomes were

made and further analysed with Southern blot and qPCR. At each time point genomic DNA was extracted and digested with HindIII (for Southern blot) or with BglI (for qPCR) enzymes. HindIII enzyme is a cellular DNA cutting enzyme thus digesting the huge DNA molecule into smaller peaces, necessary for resolving it in agarose gel. However it does not restrict HPV18 DNA, thus allowing to analyze different molecular forms of HPV18 genome stably replicating in U2OS cells.

Figure 4 represents the Southern blot analysis of stable replication of HPV18 wt and mutant genomes in U2OS cells. The replication of HPV18 genomes bearing mutations in the ORF of E4, E1^E4, E5, E6 and E7 is clearly detectable thus showing that this HPV18 mutant genomes are stably maintained in U2OS cells (lanes 5-24 in *Figure 4*). However, if both E6 and E7 proteins are mutated, HPV18 stable replication is clearly weaker than wt (compare lanes 25-28 with 1-4 in *Figure 4*). Analysing the E2C-2$^-$ and E8$^-$E2C-1$^-$ genomes replication gives us a pattern of smooth replication decrease compared to the wt HPV18. Signals from HPV18 E8$^-$ mutant also decrease in time, indicating that the expression of E8^E2 could be necessary for stable maintenance as well. The E8$^-$E2C-2$^-$ replication would be in accordance with E2C-2$^-$ and E8$^-$E2C-1$^-$ unless its last timepoint where a strong increase of replication can be noticed. The cells could have been confluent, which turns on vegetative amplification of HPV genome in U2OS cells (Geimanen et al., 2011). Watching the whole picture it is noticible that some signal bands look diffuse, what can be explained by technical errors during the purification of DNA, for example the sample was contaminated with proteins.

Southern blot analyses was carried out with uncut HPV18 DNA which allows to study different molecular forms. Markers on lines 29, 30, 47 and 48 on *Figure 4* show covalently closed circular (ccc), linear (lin) and open circluar (oc) monomeric HPV18 molecules. Replication signal for HPV18 wt and all the mutants is clearly bigger than monomeric forms, indicating that vast majority of HPV18 molecules stably replicating in U2OS cells consist of at least two HPV18 molecules, probably joined by homologous recombination (Orav et al., 2014).

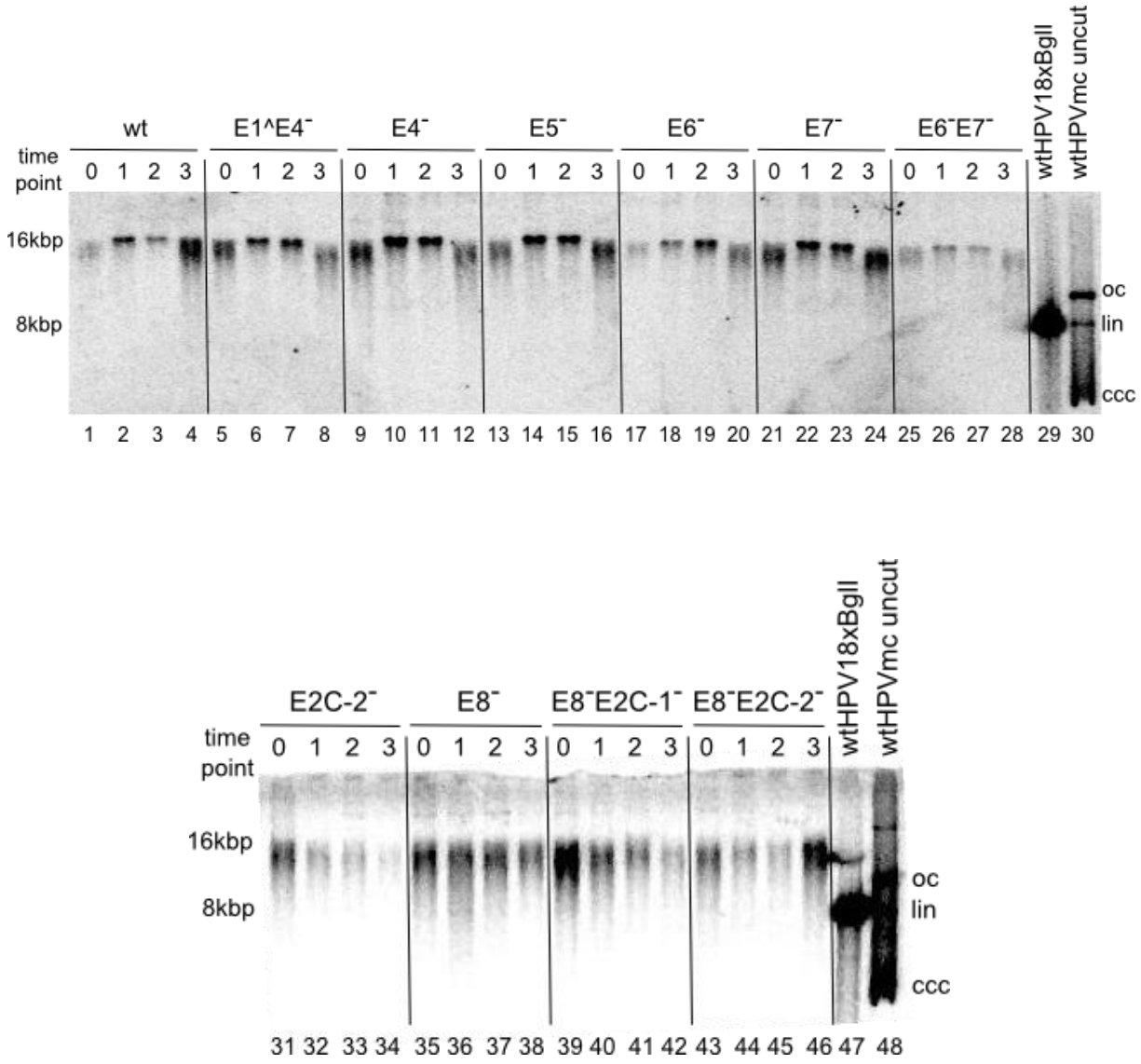

Figure 4. Stable replication analysis of HPV18 wt and its mutant genomes in U2OS cell line. HPV18 E1^E4⁻, E4⁻, E5⁻, E6⁻, E7⁻, E6⁻E7⁻, E2C-2⁻, E8⁻, E8⁻E2C-1⁻ and E8⁻E2C-2⁻ genomes with introduced pointmutations or frameshift mutations were used. The U2OS cells were transfected with 2µg of minicircles together with 1µg of the linearized pBabeNeo plasmid that brings the resistance to geniticin. The mock plate cells were transfected with MQ water for the future selection control. Cells were grown in IMDM media containing 400µg/ml G-418 antibiotic until the mock plate was empty, and drug-resistant colonies raised. After selection (timepoint 0), the cells were cultivated in subconfluent conditions and genomic DNA samples were collected after every fourth day. DNA was digested with HindIII enzyme to cleave cellular genome. The Southern blot analysis with HPV18 specific probe followed. Lines 1-28 and 31-46 represent the analysis of HPV18 wt and mutant genomes` replication signal during stable maintenance. Lines 29, 30, 47 and 48 represent the markers for linearized HPV18 mc

genome and for uncut HPV18 minicircle. From the left side it is shown the migration of open circular, linear, and supercoiled circular forms of DNA. The signal row shows the replication signal strenght as well as the migration pattern of viral DNA.

In addition to the Southern blot analysis (*Figure 4*), stable replication signals for HPV18 genomes were quantitated using qPCR. Genomic DNA was cleaved with BglI overnight and 1,25 ng of it was used for each qPCR reaction. Signals in *Table 2* are normalized against the value obtained from GAPDH gene and expressed relative to each sample 0-timepoint. Ct values from qPCR were analyzed using the comparative threshold cycle (ΔΔCt) method. Results from qPCR analyses show good correlation with Southen blot from *Figure 4*.

Table 2. qPCR analysis of HPV18 stable replication.

timepoint	0	1	2	3
genome				
HPV18wt	1,00	1,74	1,07	1,32
HPV18 E1^E4^{-}	1,00	2,14	1,52	1,00
HPV18 E4^{-}	1,00	N/D	1,41	0,87
HPV18 E5^{-}	1,00	1,74	1,41	1,41
HPV18 E6^{-}	1,00	1,32	0,87	1,07
HPV18 E7^{-}	1,00	1,32	0,93	1,52
HPV18 E6^{-}E7^{-}	1,00	1,00	0,81	1,00
HPV18 E2C-2^{-}	1,00	0,47	0,38	0,25
HPV18 E8^{-}	1,00	0,76	0,66	0,50
HPV18 E8^{-}E2C-1^{-}	1,00	0,66	1,23	0,50
HPV18 E8^{-}E2C-2^{-}	1,00	0,66	0,57	0,81

2.3.3. Analysis of E1 requirement in HPV18 stable maintenance by the E1 protein RNAi

Since HPV E1 protein is necessary in initial amplification of HPVs, stable replication of E1 mutant genome cannot be studied (Chiang et al., 1992; Frattini and Laimins, 1994; Geimanen et al., 2011; Lace et al., 2008). It has been suggested that E1 protein is dispensable for stable replication of HPV16 (Egawa et al., 2012). It was next tested whether this could be the case for HPV18 as well. One possibility is to downregulate E1 expression using siRNA-s. To test the siRNA against E1 protein (965-987nt positions in the HPV18 genome), U2OS cells were

electroporated with 2µg of HPV18 wt genome and plated onto 60mm dishes. On the third day after electroporation, 150pmol of E1 siRNA were chemically transfected to the cells using R007 reagent (Icosagen). Genomic DNA was extracted 2 and 3 days after the transfection with E1 siRNA, digested with BglI and DpnI and analysed by qPCR. 3,125ng of DNA were used for each reaction. Signals on *Figure 5* are normalized against the value obtained from GAPDH gene and expressed relative to HPV18 mock transfection 2-day timepoint. Ct values from qPCR were analyzed using the comparative threshold cycle (ΔΔCt) method.

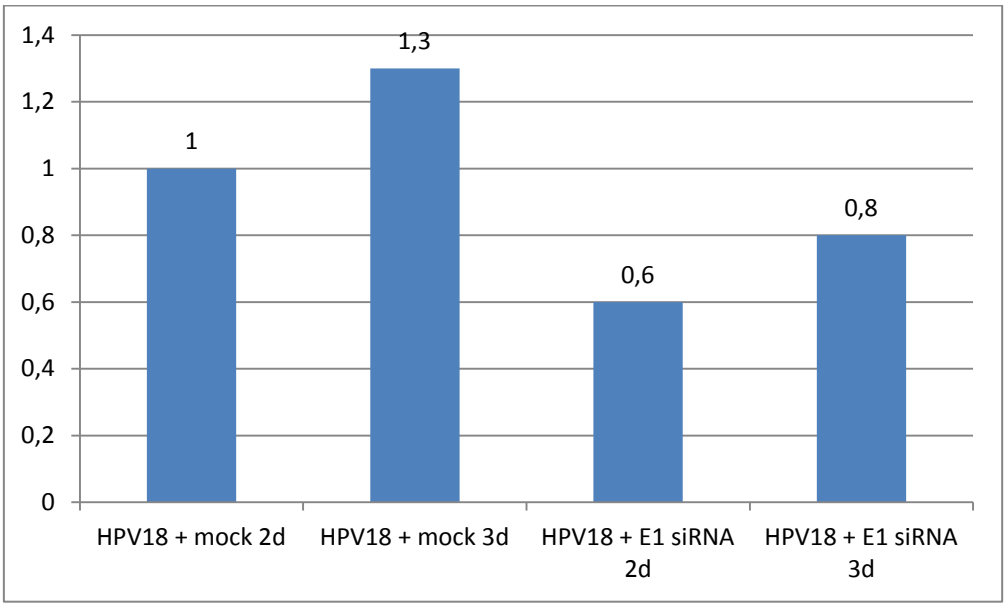

Figure 5. Effect of E1 siRNA on transient replication of HPV18. U2OS cells were electroporated with 2µg of HPV18 minicircle plasmid and plated onto 60mm dishes. 150pmol of E1 siRNA were transfected to the cells three days after electroporation using reagent R007. Genomic DNA was extracted 2 and 3 days after siRNA transfection, linearized with BglI and digested with DpnI to distinguish between replicated and transfected HPV18 DNA. Ct values from qPCR were analyzed using the comparative threshold cycle (ΔΔCt) method. Replication signals are expressed relative to HPV18 mock transfection 2-day timepoint.

As can be seen on *Figure 5*, E1 siRNA downregulates HPV18 transient replication by at least 40%. E1 protein levels during transient replication are too low to detect on Western blot analyses, so measuring transient replication is the only way to test whether this siRNA works.

The effects of E1 RNAi on stable replication of HPV18 were next analysed. U2OS cells stably maintaining HPV18 genome, derived from previous experiment shown in *Figure 4*, were plated on 60mm dishes and transfected with 150pmol of E1 siRNA (nt 965-987) by chemical transfection with R007 reagent. Genomic DNA was extracted 2 and 3 days after the transfection, digested with BglI and analysed by qPCR. 3,125ng of DNA were used for each

reaction. Signals on *Figure 6* are normalized against the value obtained from GAPDH gene and expressed relative to HPV18 pool + mock transfection 2-day timepoint. Ct values from qPCR were analyzed using the comparative threshold cycle (ΔΔCt) method.

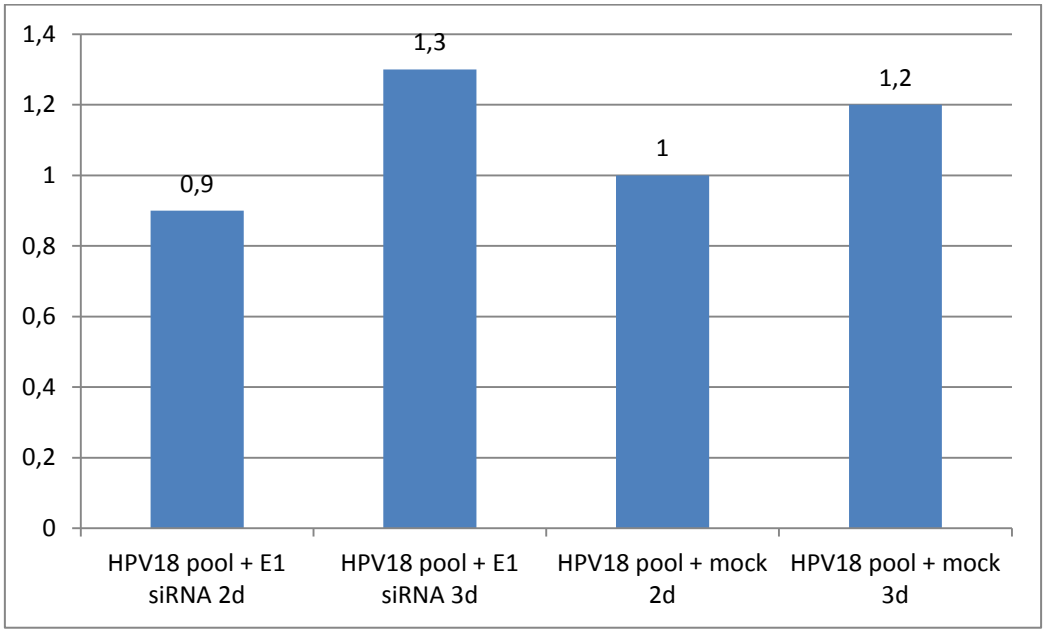

Figure 6. Effect of E1 siRNA on stable replication of HPV18. U2OS cells stably maintaining HPV18 wt genome were plated onto 60mm dishes and transfected with 150pmol of E1 siRNA by chemical transfection with R007. Genomic DNA was extracted 2 and 3 days after the transfection and cleaved with BglI. Ct values from qPCR were analyzed using the comparative threshold cycle (ΔΔCt) method. Replication signals are expressed relative to HPV18pool + mock transfection 2-day timepoint.

Effects of E1 siRNA on stable replication of HPV18 are shown in *Figure 6*. When E1 expression was decreased, a clear efect on tranisent replication occured (*Figure 5*). However knockdown of E1 seems not to interfere with stable replication of HPV18, as can be seen on *Figure 6*. These results indicate that E1 could be dispensable for stable replication of HPV18 in U2OS cells, similarly to HPV16 (Egawa et al., 2012).

2.3.4. Analysis of increased E1 expression in HPV18 stable maintenance

The U2OS cells stably maintaining HPV18 wt genome were derived from previous experiment shown in *Figure 4*, and plated on 60mm dishes for stable replication analysis. Cells were chemically transfected with 500ng or 1000ng of HPV18 E1 expression vector pM18-14. 500ng and 1000ng of both HPV18 E1 and E2 (pQMN18E2) expression vectors were added as positive controls. Expression of both E1 and E2 has to result in increase of replication as these proteins are capable of initiating replication from URR region. Empty

transfection was used for negative control and reference. First, the expression of HPV18 E1 protein was verified by Western blot analysis 24 hours after the transfection. Cells were lysed, proteins were separated in polyacrilamid gel and transfered onto membrane. Expression vector pM18-14 has HA (hemaglutinin)-tag in the N-terminus of E1 protein, so HA-tag was used to detect the expression of HPV18 E1. Beta-Tubulin serves as loading control. Signals were vizualized, and exposed to the X-Ray film (*Figure 7*).

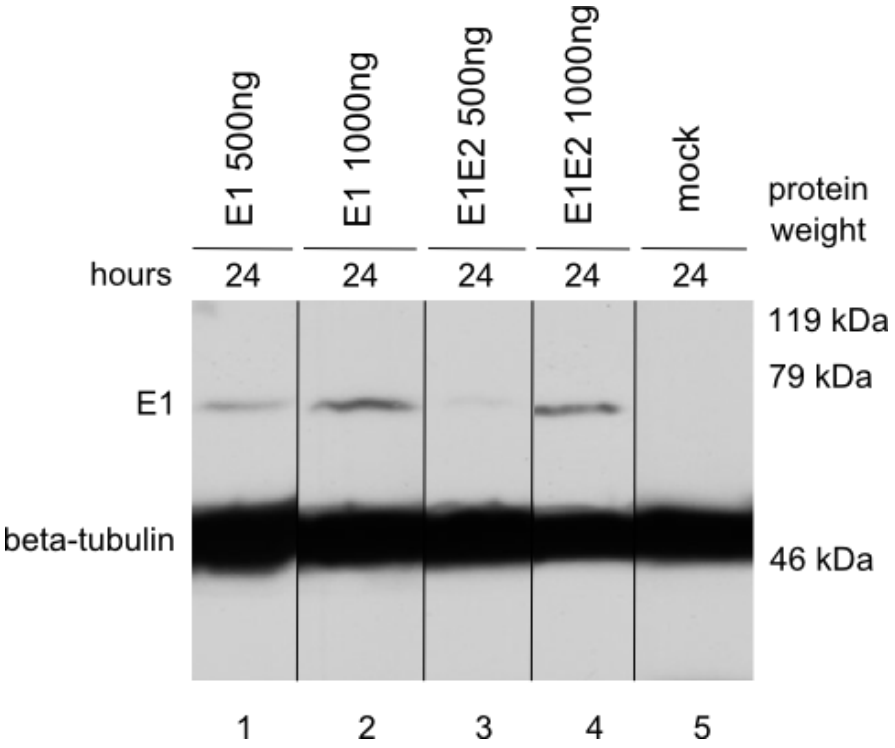

Figure 7. Western blot analysis of HPV18 E1 protein expression. U2OS cells stably maintaning HPV18 wt genome were transfected with 500ng or 1000ng of E1 and with both E1 and E2 expression vectors by chemical transfection with R007. 24 hours after the transfection cells were lysed and analysed with Western blot. Approximately 70kDa band is E1 and 50kDa protein is a loading control beta-tubulin.

Figure 7 represents the Western blot analysis of E1, and both E1 and E2 protein expression in U2OS cells stably maintaining HPV18 wt genome. The expression of E1 protein (approximately 70kDa band) is clearly detectable in a concentration-dependent manner (lanes 1 and 2, 3 and 4). Mock transfection shows the E1 signal specifity (lane 5). Expression of beta-tubulin (approximately 50kDa band) serves as loading control.

After the expression of E1 protein was verified, effect of its overexpression on stable replication of HPV18 was analysed. Genomic DNA was extracted 24 hours after transfection,

digested with BglI and analysed by qPCR. 3,125ng of DNA was used for each reaction. Signals on *Figure 8* are normalized against the value obtained from rDNA and expressed relative to mock transfection 24-hour timepoint. Ct values from qPCR were analyzed using the comparative threshold cycle (ΔΔCt) method.

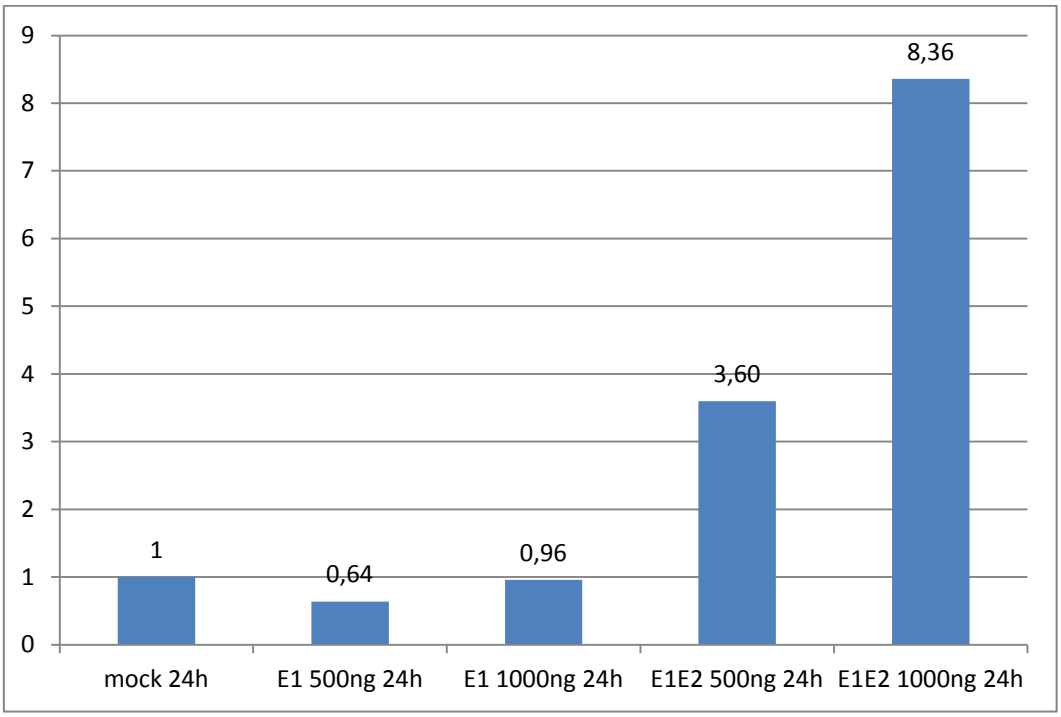

Figure 8. Effect of E1 increased expression on stable replication of HPV18 wt. U2OS cells stably maintaining HPV18 wt genome were plated onto 60mm dishes and transfected with 500ng or 1000ng of E1, and with both E1 and E2 expression vectors by chemical transfection with R007. Genomic DNA was extracted 24 hours after transfection and cleaved with BglI. Ct values from qPCR were analyzed using the comparative threshold cycle (ΔΔCt) method. Replication signals are expressed relative to the mock transfection 24-hour timepoint.

The *Figure 8* represents the replication analysis of HPV18 wt stable replication with the increased expression of E1. As expected, when transfected together, expression of E1 and E2 increases the replication of HPV18 in a concentration-dependant manner. The expression of E1 alone however does not alter the HPV18 replication to a great extent. Thus we can conclude that E1 protein is dispensable for the HPV18 stable replication. Or at least it is not limiting factor during stable mainteance. This result correlates with the previous one (*Figure 7*) as well as with the published one (Egawa et al., 2012) and indicates that at least HPV16 and HPV18 do not require E1 protein for their genome maintenance.

2.4. DISCUSSION

Human Papillomaviruses (HPVs) are the main causative agents of cervical cancer and other malignancies. Since their life cycle is strictly connected with the keratinocyte differentiation programme, it has been difficult to study their replication in vitro. Few years ago, Ustav`s lab developed a new model system for studying HPV replication (Geimanen et al., 2011). This system is based on the U2OS cell line and allows to study all three replication stages of different HPV types. Since U2OS cells are immortalized, the HPV replication functions can be studied directly. Furthermore, the HPV18, HPV5 and HPV11 gene expression in U2OS cells is identical to that observed in the natural viral infection of keratonocytes (Sankovski et al., 2014; Isok-Paas et al., unpublished data, Männik et al., unpublished data). These studies confirm that U2OS cells are suitable for studying HPVs.

The first part of the present study was to characterize the transient replication of HPV18 and to determine which gene products, apart from E1 and E2, are required for this. It has been shown before that both replication proteins E1 and E2 are necessary for the initial amplification of HPV genome (Chiang et al., 1992; Frattini and Laimins, 1994; Geimanen et al., 2011; Lace et al., 2008). Different HPV18 mutants have been constructed in a way that they do not express genes from HPV18 early region: E1^E4, E4, E5, E6, E7, E2C-2 and E8^E2 ($E8^-$). In addition HPV18 double mutants not expressing both E6 and E7, E8^E2 and E2C-1, E8^E2 and E2C-2 were constructed. Analysing initial amplification of the HPV18 mutants, it was found that E1^E4, E4, E5, E6 and E7 proteins are not required for this phase and do not alter the replication efficiancy compared to wt (*Figure 3*, lanes 1-14). The E8^E2 spliced protein is a negative regulator of viral transcription and replication. So, it was expected, that mutation in the E8^E2 will increase the replication signal, what would be in accordance with previous studies (Kurg et al., 2010; Lace et al., 2008; Stubenrauch et al., 2000). Indeed, the mutant E8 facilitates HPV18 replication to the 5-fold higher level compared with the wt HPV18 (*Figure 3*, lanes 19 and 20). Transcription analyses of HPV18 in U2OS cells revealed novel promotor P_{3000} in the ORF of E2 (Männik et al., unpublished data) that could be used to express truncated E2 proteins, containing only the C-terminal part (E2C-1 and E2C-2). It has been shown, that BPV-1 E2C acts as a regulator for transcription and replication (Lace et al., 2012). Initial amplification of HPV18 genomes bearing both mutations (in ORFs E8 and in E2C-1/E2C-2) replicate even more efficiently compared to the HPV18 genome containing only the E8- mutation (*Figure 3*, lanes 21-24). These results indicate that in addition to the main regulator E8^E2, HPV18 has two additional regulators that could control the viral copy number. E2C-2 mutant, however, caused a slight decrease in

HPV18 replication (*Figure 3*, lanes 17 nad 18). It has been shown that BPV-1 E2C, similar to HPV18 E2C-2, can, besides repressing, also activate the transcription in certain condition (Lace et al., 2012). This could be the case for HPV18 E2C-2 as well, and it would explain the approximately 40% decrease of E2C-2 mutant in initial amplification.

The second part of the present study was to characterize the HPV18 genome maintenance in U2OS cells and to determine which gene products are required for this. U2OS cells with transfected HPV18 wt and mutants were cultivated under selection for 2 weeks, after that genomic DNA samples were taken after every fourth day. It was detected that E4, E1^E4, E5, E6 and E7 mutants stably replicated during this period, thus these proteins are not required for HPV18 maintenance (*Figure 4*, lanes 5-24). However HPV18 with mutations in both E6 and E7 ORFs shows considerably lower replication signal compared to the wt (*Figure 4*, lanes 25-28). Yet this mutant replicates similarly to the wt HPV18 during intial amplification (*Figure 3*). This indicates that E6 and E7 proteins contribute to the stable maintenance of HPV18. Previous studies have also demonstrated that E6 protein is required for the HPV16, 18 and 31 to replicate extrachromosomally in the human keratinocytes and that p53 inactivation is essential for this process. It has been also shown that E6 mutant HPV16 genomes restore and increase their stable replication efficiency as p53 is inactivated in the cells (Lorenz et al., 2013; Thomas et al., 1999). So, in U2OS cells HPV18 E6 or E7 could create suitable environment for persistant viral replication. In addition to E6 and E7, HPV18 seems to need E2C-2 protein (regulator for HPV18 replication and transcription) for stable maintenance as well (*Figure 4*, lanes 31-34). Since BPV-1 E2C activates transcription (Lace et al., 2012), so could E2C-2. Perhaps it activates the expression of E2 protein, and if it is mutated, E2 transcription is decreased. This could lead to the loss of segregation function and thus HPV DNA is lost during cell division.

The HPV genome can exist in different molecular forms: monomeric and multimeric, or oligomeric. HPV oligomers are organized to a head-to-tail manner (Geimanen et al., 2011, Orav et al., 2014). The cercical carcinoma biopsy revealed, that HPV16 extrachromosomal genomes are maintained mostly as oligomers. Thus, the oligomerization of viral DNA is probably common for HPV in natural infection (Cullen et al., 1991; Kristiansen et al., 1994). As can be seen in *Figure 4*, HPV18 molecules stably replicate as molecules bigger than monomeric forms, specifically 16kb molecules. These results indicate that vast majority of HPV18 molecules stably replicating in U2OS cells consist of at least two HPV18 molecules, probably joined together by homologous recombination (Orav et al., 2014). HPVs could also integrate into the host genome. However experiments done in Ustav's lab with U2OS cells

stably maintaining HPV18 genomes have shown that vast majority of viral DNA is maintained as episomal plasmid.

The third part of present work was the analysis of E1 protein requirement during HPV18 maintenance. E1 is essential for viral genome initial as well as vegetative amplification, however it's role during stable replication of HPV18 is unclear. Previous work has been done using Cre-lox recombiantion method for deleting the E1 ORF from HPV16 genome during specific timepoints during viral life cycle. This resarch showed that for HPV16, E1 is dispensable for stable replication (Egawa et al., 2012). To analyse the importance of E1 protein during stable replication of HPV18 in U2OS cells, it's expression was downregulated with specific siRNA. As can be seen in *Figure 6*, downregulation of E1 protein does not alter the stable replication of HPV18. However replication inhibiton occured when using siRNA during intial amplification (*Figure 5*). In addition to downregulation, E1 expression was also increased during stable replication of HPV18. Two expression vectors were transfected into U2OS cells stably replicating HPV18: E1 and E1+E2 together. In case of E1 and E2 expression, clear rise in replication signal occured. However by increasing the expression of E1 protein without E2, no changes in the replication could be detected (*Figure 8*). These results do not give absolute certainty whether E1 is necessary for stable maintenace or not. But they show that it is not a limiting factor during the stable replication of HPV18 in U2OS cells. However it in unlikely that there would be an excess of E1 protein in the cells. E1 is capable of activating DDR, probably by inducing double-stranded DNA breaks into the viral as well as the host genome (Reinson et al., 2013; Sakakibara et al., 2011). If E1 is dispensable during maintenance phase, HPV replication relies only on cellular proteins. E2 protein could be the only viral protein used during stable replication and it is responsible for correct partitioning of viral genome between daughter cells. It could also, together with E2C-2 protein, regulate viral transcription (Kurg et al., 2010).

Next stages of this study should concentrate on cellular factors responsible for stable replication of HPV18. Furthermore, it is unclear which mechanisms HPV uses during this replication stage. The exact roles of E6, E7 and E2C-2 in stable replication phase needs to be studied as well.

CONCLUSIONS

The aim of this thesis was to chracterize the roles of HPV18 early proteins during initial amplification and stable maintenance in U2OS cells. For this purpose all known early genes were mutated alone or in combination with each other in HPV18 genome. These mutant genomes were intoduced into U2OS cells, and first, their necessity in initial amplification was studied. It was shown that, besides E1 and E2, none of the other early proteins are essential in HPV18 initial amplification. However, two additional regulators for HPV18 replication and transcription, besides the known E8^E2, were described: E2C-1 and E2C-2. These regulators consist of C-terminal part of E2 protein and could be similar to the BPV-1 E2C. Next, the transfected cells were selected and passaged to study the role of early genes of HPV18 during stable replication. For efficient stable replication, HPV18 need either E6 or E7 expression, the double mutant where expression of both of these proteins is eliminated, showed considerably lower replication level. In addition to E6 and E7 expression, E2C-2 expression seems to be essential for stable maintenance. It could be required for transcription activation from HPV18 promotors.

Second part of the study focused on E1 protein and its potential role during stable replication of HPV18. Neither downregulation by RNAi nor overexpression altered the levels of HPV18 stably replicating in U2OS cells. This indicates that either HPV18 E1 is dispensable for stable replication, as shown for HPV16, or is required at very low concentrations. Thus, stable replication of HPV18 may rely solely on cellular factors.

The further work would be the study of the cellular mechanisms and cellular factors involved in HPV18 stable replication. Also, it would be reasonable to analyze the roles of HPV18 early proteins during the last replication stage – vegetative amplification.

Uurimus inimese papilloomiviiruse tüüp 18 stabiilsest säilumisest.

Tatjana Tsõpova

Resümee

Inimese papilloomiviirused (HPV) on väikesed dsDNA viirused, mis on väga laialdaselt levinud. Neid on leitud nii imetajatelt, lindudelt kui ka roomajatelt. Nad nakatavad läbi mikrohaavandite naha ning limaskestade epiteelkudesid ning põhjustavad kondüloome ja ka soolatüükaid, infektsioon möödub tavaliselt umbes 24 kuuga. Mõningatel juhtudel muutub aga infektsioon persistentseks ning sellest võib areneda vähkkasvaja. Kõige sagedamini põhjustab HPV emakakaelavähki, hinnanguliselt põhjustab see 270 000 surmajuhtumit aastas.

HPV-de replikatsioon toimub keratinotsüütides ning see jagatakse kolmeks faasiks. Esmane amplifikatsioon toimub peale nakatamist ning selle käigus viiakse viiruse genoomi koopiaarv 50-100 koopiani raku kohta. Järgneb stabiilne säilumine, mille käigus toimub viiruse replikatsioon üks kord rakutsükli jooksul ning koopiaarv püsib konstantne. Kolmas etapp, vegetatiivne või hiline amplifikatsioon, leiab aset juba terminaalselt differentseerunud keratinotsüütides ning selle käigus tõuseb viiruse genoomi koopiaarv mitme tuhandeni raku kohta. Vegetatiivsele amplifikatsioonile järgneb viiruse DNA pakkimine kapsiidi ning uute viiruste vabanemine.

Selle töö eesmärgiks oli uurida HPV tüüp 18 varajaste valkude vajalikkust esmase amplifikatsiooni ja stabiilse säilumise etappides. Selleks kasutati U2OS rakkudel põhinevat mudelsüsteemi, mis on sobilik mitmete nahka ning limaskestade epiteelkudesid nakatavate HPV tüüpide replikatsiooni uurimiseks.

Leiti, et peale põhiliste replikatsioonivalkude E1 ja E2, ei vaja HPV18 teisi varajasi viirusvalke esmase amplifikatsiooni läbiviimiseks. Lisaks teadaolevale E8^E2 valgule identifitseeriti ka kaks uut regulaatorvalku, mis sisaldavad ainult E2 valgu C-terminaalset osa. HPV18 võib neid kasutada oma genoomi koopiaarvu kontrolliks.

Stabiilse säilumise uurimisest selgus, et selle efektiivseks toimumiseks on vaja kas E6 või E7 valku, sest viiruse genoom, kus mõlemad valgud olid muteeritud, jäi püsima oluliselt madalamal tasemel. Lisaks selgus, et vajalik on ka E2C-2 valk, mis võib teatud tingimustel aktiveerida transkriptsiooni viiruse promootoritelt. Uuriti ka E1 valgu rolli stabiilse replikatsiooni käigus. Tulemustest selgus, et ei E1 valgu mahareguleerimine RNA interferentsiga ega ka E1 valgu üleekspressioon ei mõjuta HPV18 stabiilset replikatsiooni.

See võib tähendada, et E1 valku ei ole stabiilseks säilumiseks vaja ning viirus kasutab selles etapis ainult rakulisi valke oma genoomi replitseerimiseks. Samuti võib see näidata, et E1 valku on rakus ülehulgas ning see ei ole limiteeriv komponent HPV18 stabiilses replikatsioonis.

Edasised uuringud peaksid keskenduma rakuliste valkude, mis osalevad HPV stabiilses replikatsioonis, identifitseerimisele ning nende võimaliku rolli kindlaks määramisele. Samuti tuleks välja selgitada, millesed viiruse varajased valgud on vajalikud vegetatiivses amplifikatsioonis.

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